J. Antibiot. 58(12): 759–765, 2005

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Biosynthesis of Indocarbazostatin B, Incorporation of D-[*U*-^{13}C] Glucose and L-[2-^{13}C] Tryptophan

Yufei Feng, Shinya Mitsuhashi, Takao Kishimoto, Makoto Ubukata

Received: September 9, 2005 / Accepted: November 19, 2005

Abstract High incorporation of D-[*U*-^{13}C] glucose and L-[*indole*-2-^{13}C] tryptophan into indocarbazostatin B (**2**) was observed in a biosynthetic study using a mutant strain, *Streptomyces* sp. MUV-7-8. The original strain, *Streptomyces* sp. TA-0403 produced a small amount of indocarbazostatin (**1**) and indocarbazostatin B (**2**), which displayed potent biological activities. To facilitate biosynthetic studies, we selected high indocarbazostatin producing mutant strains. The first mutants, *Streptomyces* sp. MUV-6-83 and MUV-6-17, produced indocarbazostatins C (**3**) and D (**4**) as well as **1** and **2**. When the production medium was supplemented with D-tryptophan, the MUV-6-17 mutant produced K252c (**5**), whereas when L-tryptophan was added, it produced K252d (**6**). On further UV treatment of the mutant strain MUV-6-83, we finally obtained a new mutant producer, *Streptomyces* sp. MUV-7-8, that produced **2** as a major metabolite with higher productivity. This mutant producer enabled us to do a feeding experiment of the envisioned precursors, glucose and tryptophan.

Keywords indolocarbazol antibiotics, biosynthesis, benzil-benzilic acid rearrangement, indocarbazostatin, ^{13}C–^{13}C coupling

Introduction

We were able to discover indocarbazostatin (**1**) and indocarbazostatin B (**2**) as novel inhibitors of NGF-induced neuronal differentiation in PC12 cells [1~3]. The productivities of **1** and **2** were 1.2 mg and 0.9 mg, respectively, from 35 liters of a culture broth of the original producer, *Streptomyces* sp. TA-0403. The biological activity of **1** was more potent than that of a known inhibitor, K252a [4]. There was some difference between indocarbazostatins and other known indolocarbazole antibiotics in their molecular shape, because of the opposite absolute configurations at C-3′ of all of the indocarbazostatins and the atropisomeric chirality in the 7b~7c axes of **2** and **4** [3, 5]. To approach the biosynthetic study of these unique compounds, we decided to treat the original producer by repeated UV irradiation. After irradiation with UV six times, we obtained *Streptomyces* sp. MUV-6-83 and MUV-6-17 that produced new analogs, indocarbazostatins C (**3**) and D (**4**) together with **1** and **2** [5]. Since instability was observed in the productivity of the mutant, the incorporation experiment of a labeled precursor failed. The improved mutant producer, *Streptomyces* sp. MUV-7-8, was obtained by another UV treatment of *Streptomyces* sp. MUV-6-83. The new mutant showed high specificity in the productivity of **2** as a major metabolite that is easy to separate. In this paper, we report the precise selection procedure of the producer of indocarbazostatins, identification of K-252c (**5**) and K-252d (**6**) [6] in a broth of a mutant producer, control of metabolite production by adding tryptophan, incorporation of putative precursors, D-[*U*-$^{13}C_6$] glucose and L-[*indole*-2-^{13}C] tryptophan into **2** using a new mutant strain, *Streptomyces* sp. MUV-7-8 (Fig. 1).

M. Ubukata (Corresponding author), **Y. Feng, S. Mitsuhashi, T. Kishimoto:** Graduate School of Agriculture, Hokkaido University, North-9, West-9, Kita-ku, Sapporo 060-8589, Japan, E-mail: m-ub@for.agr.hokudai.ac.jp

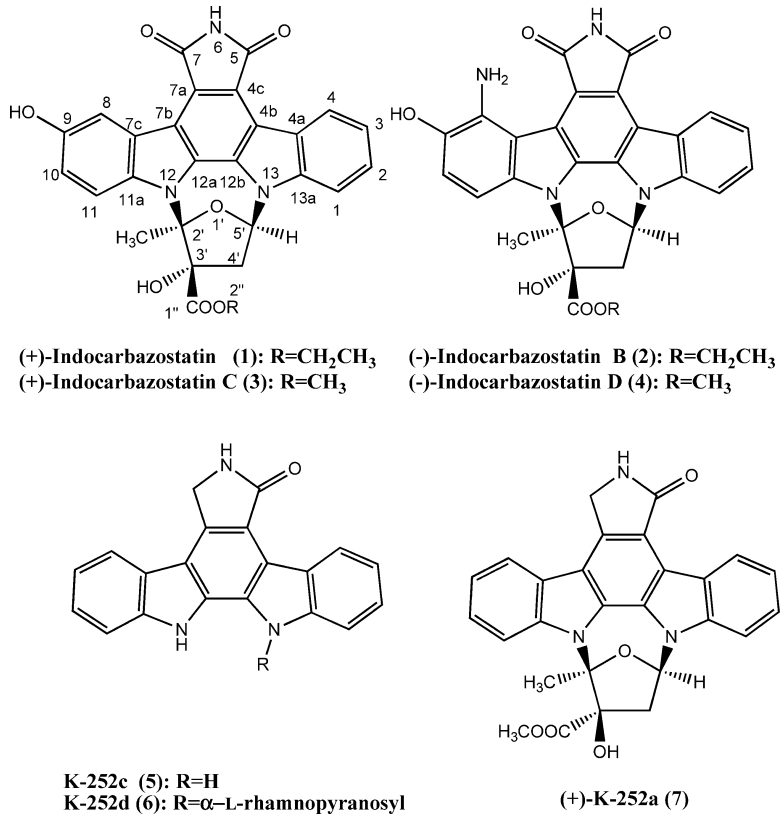

Fig. 1 Structures of indocarbazostatins and their related compounds.

Material and Methods

Chemicals

L-[*indole*-2-^{13}C] Tryptophan (98 atom % ^{13}C) and D-[*U*-$^{13}C_6$] glucose (99 atom% ^{13}C) were purchased from Cambridge Isotope Laboratories Inc. (USA) and Isotec Inc. (USA), respectively.

Microorganism

The original strain *Streptomyces* sp. TA-0403 [2] was a generous gift from Dr. A. Kawashima and Mr. T. Andoh of Taisho Pharmaceutical Co. Ltd.

Isolation of K252c (5) and K252d (6) from a Culture Broth of a Mutant *Streptomyces* sp. MUV-6-17 Fermented in the Presence of L-Tryptophan or D-Tryptophan

A slant culture of the mutant, *Streptomyces* sp. MUV-6-17, was inoculated into twenty 500 ml K-1 flasks each containing 50 ml of the culture medium consisting of glucose 0.5%, soluble starch 2%, NZ Case (Humko Sheffield Chemical Co.) 0.3%, yeast extract 0.3%, fish meal 0.5%, $CaCO_3$ 0.2% (pH 6.5 before sterilization). The flasks were incubated at 30°C for 2 days on a rotary shaker (200 rpm). The seed culture (2 ml) was transferred into a hundred 500 ml K-1 flasks each containing 50 ml of the production medium consisting of glucose 0.43%, soluble starch 2.17%, soybean meal 0.59%, corn steep liquor 0.59%, Pharmamedia 1.18%, yeast extract (Difco Laboratories) 0.2%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3%, and Diaion HP-20 (Mitsubishi Chemical Co.) 1%, L-tryptophan 0.1% (pH 7.0). Another seed culture (2 ml) was transferred into a hundred 500 ml K-1 flasks each containing 50 ml of the production medium consisting of the above components except for addition of D-tryptophan instead of L-tryptophan. Both fermentations were carried out at 30°C for 4 days on a rotary shaker. The combined culture was centrifuged into mycelium and supernatant, and the mycelium was extracted with 10 liters of acetone. The acetone extract was partitioned into $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated *in vacuo*, and the residue was subjected to silica gel column chromatography (8 cm i.d.×20 cm, silica gel 40 μm, $CHCl_3 : CH_3OH = 20 : 1$) followed by reversed-phase column chromatography (3 cm i.d.×20 cm, Cosmosil 40 C_{18}-PREP,

eluent: 60% CH_3OH) and preparative HPLC (2 cm i.d.×25 cm, Mightysil RP-18, eluent: 50% CH_3CN) to yield 1.76 mg of K252c (**5**) and 1.27 mg of K252d (**6**).

5: FAB-MS: *m/z* 312 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 4.95 (2H, br s), 7.20 (1H, br t), 7.29 (1H, br t), 7.43 (1H, br t), 7.45 (1H, br t), 7.69 (1H, d, *J*=8.05 Hz), 7.76 (1H, d, *J*=8.05 Hz), 8.03 (1H, d, *J*=7.8 Hz), 8.46 (1H, br s), 9.19 (1H, d, *J*=7.7 Hz), 11.57 (1H, br s), 11.75 (1H, br s). **6**: FAB-MS: *m/z* 459 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 1.68 (3H, d, *J*=7.1 Hz), 4.03 (1H, br t), 4.16 (1H, br q), 4.48 (2H, m), 4.98 (2H, br s), 5.00 (1H, br), 5.43 (1H, br), 6.73 (br s), 7.24 (1H, br t), 7.31 (1H, br t), 7.46 (1H, br t), 7.49 (1H, br t), 7.60 (1H, d, *J*=8.5 Hz), 7.68 (1H, d, *J*=8.3 Hz), 8.05 (1H, d, *J*=7.7 Hz), 8.54 (1H, br s), 9.45 (1H, d, *J*=7.5 Hz), 11.68 (1H, br s).

Isolation of ^{13}C-Labeled Indocarbazostatin B (8) from a Culture Broth of *Streptomyces* sp. MUV-7-8 Fermented in the Presence of [*U*-$^{13}C_6$] Glucose

A slant culture of the mutant, *Streptomyces* sp. MUV-7-8, was inoculated into ten 500 ml K-1 flasks each containing 70 ml of culture medium consisting of glucose 0.5%, soluble starch 2%, NZ Case (Humko Sheffield Chemical Co.) 0.3%, yeast extract 0.3%, fish meal 0.5%, $CaCO_3$ 0.2% (pH 6.5 before sterilization). The flasks were incubated at 30°C for 2 days on a rotary shaker (200 rpm). The seed culture (2 ml) was transferred into twenty 500 ml K-1 flasks each containing 100 ml of the production medium consisting of glucose 0.43%, soluble starch 2.17%, soybean meal 0.59%, corn steep liquor 0.59%, Pharmamedia 1.18%, yeast extract (Difco Laboratories) 0.2%, NaCl 0.3%, 0.05%, $CaCO_3$ 0.3%, and Diaion HP-20 (pH 7.0). One day after fermentation, 1% of D-[*U*-$^{13}C_6$] glucose was added to each flask. The culture was continued for another 4 days. The culture broth was centrifuged into mycelium and supernatant, the mycelium was extracted with acetone to give 5 g of extract. The acetone extract was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated *in vacuo* to give 1.2 g of a syrup, the residue was subjected to silica gel column chromatography (5 cm i.d.×10 cm, Silica Gel 60N, hexane : acetone=3 : 2) to afford 41.3 mg of crude indocarbazostatins. The crude sample was separated on a HPLC column (2 cm i.d.×25 cm, Mightysil RP-18) eluting with 75% CH_3OH to yield 5.4 mg of Mars yellow syrup. The crude material was crystallized from acetone to give 3.8 mg of purified ^{13}C-labeled indocarbazostatin B (**8**). The NMR measurements of **8** were carried out in DMSO-d_6 using a 2.5 mm NMR micro tube (WILMAD, 520-1A for Bruker microprobe).

Isolation of ^{13}C-Labeled Indocarbazostatin B (9) from a Culture Broth of *Streptomyces* sp. MUV-7-8 Fermented in the Presence of L-[*indole*-2-^{13}C] Tryptophan

The seed culture (2 ml) as noted above was transferred into twenty 500 ml K-1 flasks each containing 100 ml of the production medium consisting of glucose 0.43%, soluble starch 2.17%, soybean meal 0.59%, corn steep liquor 0.59%, Pharmamedia 1.18%, yeast extract (Difco Laboratories) 0.2%, NaCl 0.3%, $CaCO_3$ 0.3%, and Diaion HP-20 (pH 7.0). One day after fermentation, 0.05% of L-[*indole*-2-^{13}C] tryptophan was added to each flask. The culture was continued for another 4 days. The culture broth was centrifuged into mycelium and supernatant, and the mycelium was extracted with acetone to give 6 g of extract. The acetone extract was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated *in vacuo* to give 1.1 g of a syrup, which was subjected to silica gel column chromatography (5 cm i.d.×10 cm, Silica Gel 60N, hexane : acetone=3 : 2) to afford 29.0 mg of crude materials. The crude sample was purified on a HPLC column (2 cm i.d.×25 cm, Mightysil RP-18) eluting with 75% CH_3OH to yield 5 mg of Mars yellow syrup. The syrup was crystallized from benzene to give 1.7 mg of purified ^{13}C-labeled indocarbazostatin B (**9**) and 2.2 mg of a residue from the mother liquor. NMR measurements of **9** were done in DMSO-d_6 using a 2.5 mm symmetrical micro tube (SHIGEMI, DMS-0025).

Instruments for Structural Determination

Optical rotation was measured with a Horiba SEPA-300 spectrometer. UV spectra were recorded on a BECKMAN DU-600 spectrophotometer, ^1H NMR spectra were measured on a JEOL JNMEX-270, a JNM-La400, a Bruker AMX-500 spectrometers, and MS spectra were recorded on a JEOL JMS-AX500 instrument. Assignment of ^{13}C NMR of **2** in DMSO-d_6 is based on ^1H-^1H COSY, HMQC and HMBC analyses, and the data of ^{13}C-^{13}C coupling constants in **8** and **9**.

Results and Discussion

Mutation of *Streptomyces* sp. TA-0403 and Production of K252c (5) and K252d (6)

The spore of *Streptomyces* sp. TA-0403 [2] was obtained after 16-day culture on GYM agar plate (glycerol 0.1%, glucose 0.1%, yeast extract 0.1%, oatmeal 2%, agar 1.8%) at 30°C. The spore suspensions (1.0×10^7, 1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3 spore/ml) were irradiated with UV light (253.7 nm) for 0, 15, 30, 45, 60, 75 and 90 seconds at the distance of 25 cm under the UV lamp. Single

spore isolation was performed using the plates that showed lethal rates of which were around 99.91～99.95%. Two hundred single colonies were picked after three days incubation on SK#2 agar plate (soluble starch 2%, glucose 0.5%, yeast extract 0.5%, bactopeptone 0.5%, meat extract 0.3%, KH_2PO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.06%, agar 2%, pH 7.6). A high-producing mutant was selected and mutated again using the same method. After 6 times repeated mutation of each isolated mutant, we obtained a high-producing mutant, *Streptomyces* sp. MUV-6-17, that produced other indocarbazole antibiotics, as well as a high-producing mutant MUV-6-83 that showed slightly different HPLC profile. In the case of the mutant MUV-6-17, addition of 0.1% D-tryptophan in the production medium remarkably enhanced the productivities of **1**, **3** and peaks at 21 minutes on the HPLC (Mightysil RP-18, 4.6 mm i.d.×250 mm, 0.6 ml/minute, solvent A: CH_3CN 30～70%; B: 0.1% CH_3COOH; linear gradient: 0～40 minutes; 40°C), and slightly enhanced those of **4** and a peak at 19 minutes but not **2**, whereas L-tryptophan dominantly enhanced the productivities of **1** and a peak at 19 minutes on the HPLC, enhancements of **2**, **3** and **4** were weak in this case. The two peaks at 19 minutes and 21 minutes showed characteristic UV absorption patterns of an indolo[2,3-a]pyrrolo[3,4-c]carbazole-5(6*H*)-one system. We therefore decided to isolate these compounds and identified the peaks at 21 minutes and 19 minutes to be K252c (**5**) and K252d (**6**), respectively (Table 1). Time course of the productivities of these compounds showed that **5** and **6** did not shift to **1**～**4** and did not disappear in even 9 days' cultivation (data not shown). These data suggested that **5** and **6** may be shunt products but not direct precursors of indocarbazostatins.

Table 1 Additive effects of tryptophan on the productivities of indocarbazostatins and their related compounds.

Comp. / Additive	Relative productivity					
	6	**5**	**3**	**4**	**1**	**2**
None	n.d.	1.4	n.d.	n.d.	1	4.6
D-Tryptophan	0.7	9.8	18.9	3.4	11.1	5.3
L-Tryptophan	11.3	3.0	2.1	1.1	32.8	7.8

The relative productivity was calculated from the area ratio of each peak to the peak area of incocarbazostatin (**1**) on HPLC profile (Mightysil RP-18, solvent A: CH_3CN 30～70%; B: 0.1% CH_3COOH; linear gradient: 0～40 minutes). The retention times of **6**, **5**, **3**, **4**, **1** and **2** were 19, 21, 24, 25, 26, 27 minutes respectively.

Incorporation of D-[*U*-$^{13}C_6$] Glucose into Indocarbazostatin B (2)

To approach a biosynthetic study of indocarbazostatins, we carried out further mutation of *Streptomyces* sp. MUV-6-83 by UV irradiation. A new mutant producer, *Streptomyces* sp. MUV-7-8 which dominantly produced indocarbazostatin B (**2**), was inoculated into 500-ml K-1 flasks each containing 70 ml of the seed medium. After 2 days' culture, the seed culture (2 ml) was transferred into twenty 500 ml K-1 flasks each containing 100 ml of the production medium without tryptophan and $MgSO_4 \cdot 7H_2O$, because the productivity of **2** redoubled without the Mg ion and was not affected by addition of L, D or D,L-tryptophan. One day after fermentation, 1% of D-[*U*-$^{13}C_6$] glucose was added to each flask. The culture was continued for another 4 days. The culture broth was centrifuged into mycelium and supernatant, the mycelium was extracted with acetone. The acetone extract was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated *in vacuo* to give a syrup, which was subjected to silica gel column chromatography (hexane : acetone = 3 : 2) to afford 41.3 mg of crude materials. The crude sample was separated on a HPLC column (Mightysil RP-18) eluting with 75% CH_3OH to yield 5.4 mg of Mars yellow syrup. The syrup was crystallized from acetone to give purified ^{13}C-labeled indocarbazostatin B (**8**). Incorporation of D-[*U*-$^{13}C_6$] glucose into the sugar moiety of **8** was determined by 1D-INADEQUATE experiment, which revealed the direct incorporation of D-[*U*-$^{13}C_6$] glucose. In the same experiment, incorporation of D-[*U*-$^{13}C_6$] glucose into the aglycon moiety was also observed. L-Tryptophan would be derived from glucose through the shikimic acid pathway. To avoid such randomization of labeled glucose as in the case of biosynthetic studies of staurosporine [7], the precursor was added to the culture one day after inoculation that starts the accumulation of **2**. The result afforded characteristic ^{13}C-^{13}C couplings at C-7b–C-12a, and C-4b–C-12b but not at C-7–C-7a, C-7c–C-11a, C-8–C-9–C-10–C-11, C-4c–C-5, C-4a–C-13a, C-1–C-2–C-3–C-4 in **8** (Table 2, Fig. 2). The labeled three-carbon unit, C-3–C-4–C-5 of the ribosyl moiety would be lost as glyceraldehyde-3-phosphate in the tryptophan biosynthesis [8] as shown in Fig. 2. Thus glucose is precursor of the sugar moiety of indocarbazostatins and L-tryptophan is that of aglycon of **2** as in the case of staurosporine.

Incorporation of L-[*indole*-2-^{13}C] Tryptophan

Incorporation experiment of L-[*indole*-2-^{13}C] tryptophan was achieved in a similar manner of the above D-[*U*-$^{13}C_6$] glucose feeding experiment using *Streptomyces* sp. MUV-7-8. The seed culture (2 ml) as noted above was transferred

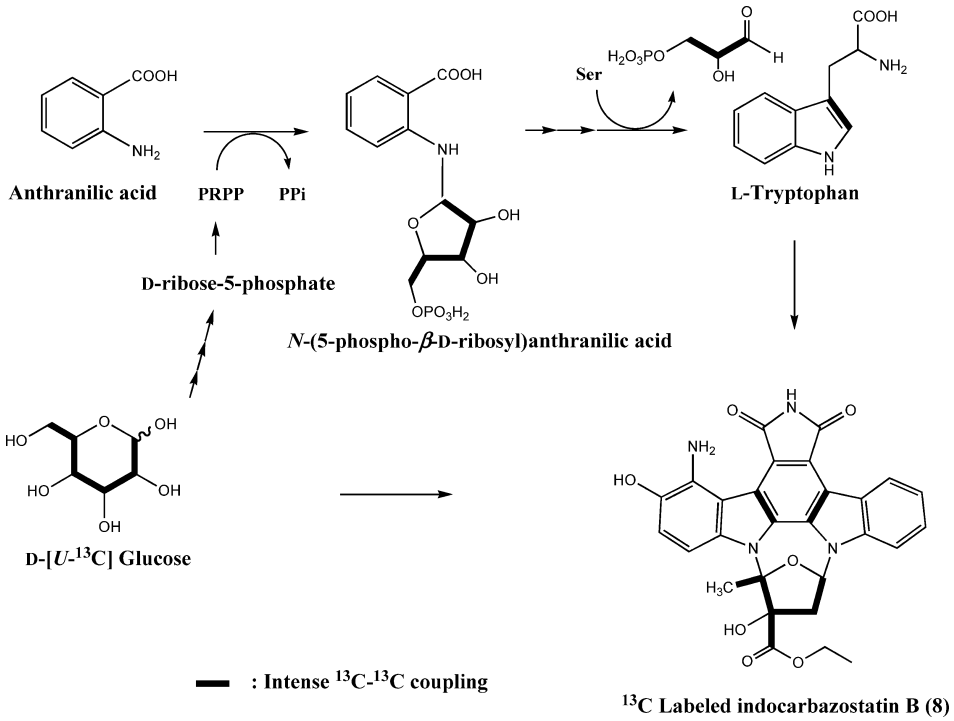

Fig. 2 Direct and indirect incorporations of D-[*U*-^{13}C] glucose into ^{13}C labeled indocarbazostatin B (**8**).

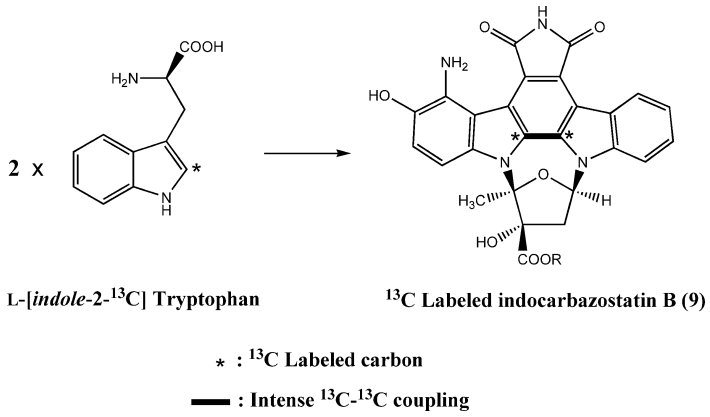

Fig. 3 Incorporation of L-[*indole*-2-^{13}C] tryptophan into ^{13}C labeled indocarbazostatin B (**9**).

into twenty 500 ml K-1 flasks each containing 100 ml of the production medium modified to be free of tryptophan and $MgSO_4$. One day after fermentation, 0.05% of L-[*indole*-2-^{13}C] tryptophan was added to each flask. The culture was continued for another 4 days. The culture broth was centrifuged into mycelium and supernatant, the mycelium was extracted with acetone to give a crude extract, which was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated *in vacuo* to give a residue, which was subjected to silica gel column chromatography (hexane : acetone = 3 : 2) to afford crude materials. Purification of the crude materials was carried out on a HPLC column (Mightysil RP-18) eluting with 75% CH_3OH to yield 5 mg of a Mars yellow syrup. The syrup was crystallized from benzene to afford 1.7 mg of purified ^{13}C-labeled indocarbazostatin B (**9**). The ^{13}C NMR of **9** showed the distinctly enriched signals at C-12a (relative intensity 17.7) and C-12b (relative intensity 11.5) with a couple of strong doublet signals ($J_{12a,12b}$ = 76.5 Hz) as shown in Table 2 and Fig. 3, thereby establishing that **9** was

produced from two intact tryptophan units as in the case of staurosporine biosynthesis [9] (Fig. 4).

The Proposal Biosynthetic Pathway of Indocarbazostatins in *Streptomyces* sp. MUV-7-8

The structure units, a pyranose derived from glucose and aglycon derived from two tryptophan units, would be connected between the N-13 position and the anomeric position at C-5′ by *N*-glycosyltransferase. Another C–N bond formation might be catalyzed by an oxidase such as P450 homolog as in the case of staurosporine biosynthesis [10~12]. The putative 1,2-diketone (A) would rearrange to furanose-type structure (B) by stereoselective benzil-benzilic acid rearrangement. This type of benzil-benzilic acid rearrangement would thermodynamically afford K-252a type compound but not C-3′-epi compounds such as indocarbazostatins **1**~**4** as shown in the synthetic study of K-252a [13]. Thus critical oxidation reaction would occur to give 1,2-diketone which leads to stereoselective ring contraction in the same active site of the oxidase, and this unique oxidase would have a 3-dimensional structure in the active site that makes it possible to construct the thermodynamically unfavorable structure to give hindered indocarbazostatins (Fig. 4). Recently, increasing amount of information has become available on the molecular genetics of indolocarbazole biosynthesis [14~17]. Cloning of a biosynthetic gene cluster of indocarbazostatins in *Streptomyces* sp. MUV-7-8 might clarify this unique oxidase homolog as well as the monoamineoxygenase homolog that constructs the atropisomeric chirality in the 7b~7c axis of indocarbazostatin B (**2**).

In conclusion, attempts to enhance productivity of indocarbazostatins by mutating the original producer, *Streptomyces* sp. TA-0403, with UV irradiation, led to acquirement of a high-producing mutant, *Streptomyces* sp. MUV-6-83 and MUV-6-17. *Streptomyces* sp. MUV-6-17 produced K-252c (**5**) in the presence of D-tryptophan, whereas K-252d (**6**) was produced in the presence of L-tryptophan in the production medium. Although L-tryptophan improved the productivity of indocarbazostatin

Table 2 ^{13}C NMR assignments for ^{13}C labeled indocarbazostatin B, **8** and **9** (DMSO-d_6)[a]

Position	^{13}C	**8**	**9**
		J_{cc}, Hz	
1	109.9	—	—
2	127.2	—	—
3	121.1	—	—
4	124.9	—	—
4a	121.3	—	—
4b	115.6	$J_{4b,12b}$=59	—
4c	120.5[c]	—	—
5	170.2[d]	—	—
7	169.6[d]	—	—
7a	128.3[c]	—	—
7b	111.8	$J_{7b,12a}$=60	—
7c	115.9	—	—
8	132.8	—	—
9	148[b]	—	—
10	118.8	—	—
11	118.3	—	—
11a	132.2	—	—
12a	130.6	$J_{12a,7b}$=60	$J_{12a,12b}$=76.5
12b	127.2	$J_{12b,4b}$=59	$J_{12b,12a}$=76.5
13a	137.8	—	—
2′	103.1	$J_{2',2'Me}$=45.5, $J_{2',3'}$=39	—
2′-Me	23.1	$J_{2'Me,2'}$=45.5,	—
3′	84.4	$J_{3',2'}$=39, $J_{3',1''}$=64, $J_{3',4'}$=38	—
3′-OH	—	—	—
4′	44.2	$J_{4',5'}$=36, $J_{4',3'}$=38	—
5′	85.5	$J_{5',4'}$=36	—
1″	169.7	$J_{1''\sim3'}$=64	—
2″	61.7	—	—
3″	12.7	—	—

[a] 125 MHz for ^{13}C NMR and 500 MHz for ^{1}H NMR.
[b] Detected by HMBC spectrum.
[c, d] Assignments may be interchangeable.

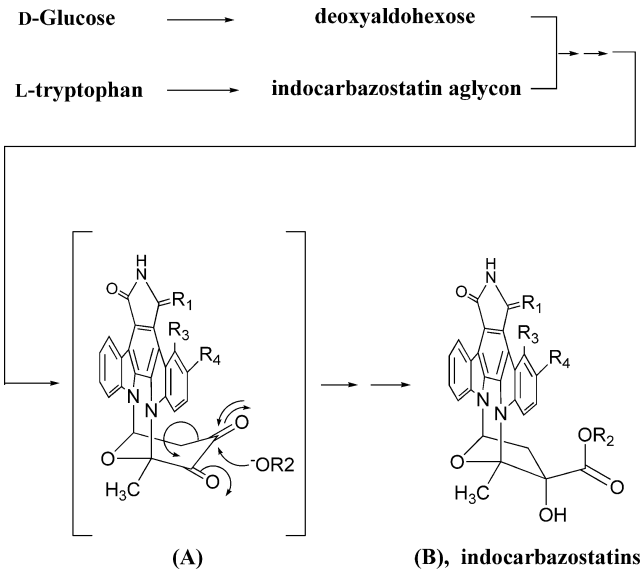

Fig. 4 Putative transformation of sugar moiety in indocarbazostatin biosynthesis.

(**1**) and **6**, and D-tryptophan enhanced the productivities of **1**, **3** and **5**, additive effects of L- or D-tryptophan in the productivities of **2** and **4** were much less than those of the other metabolites. The addition of Diaion HP-20 was effective for the production of indocarbazostatins and its related compounds. We next focused our attention on a biosynthetic study of indocarbazostatins, and obtained a suitable mutant, *Streptomyces* sp. MUV-7-8, for this purpose. *Streptomyces* sp. MUV-7-8 mainly produced **2**, which was hardly affected by tryptophan addition. D-[*U*-^{13}C] Glucose was directly incorporated into the sugar moiety and indirectly incorporated in aglycon of **2**, whereas L-[*indole*-2-^{13}C] tryptophan was directly incorporated in aglycon of **2** to give ^{13}C labeled indocarbazostatin B, **8** and **9**, respectively in these feeding experiments using this mutant. The final assignment of ^{13}C NMR of indocarbazostatin B in DMSO-d_6 shown in Table 2 is based on ^{1}H–^{1}H COSY, HMQC and HMBC analyses of **2**, and the data of ^{13}C–^{13}C coupling constants in **8** and **9**.

Acknowledgment This study was supported in part by a Grant-in Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors are grateful to Dr. E. Fukushi (Section of GC-MS NMR measurement, Hokkaido University) for NMR measurements.

References

1. Ubukata M, Tamehiro N, Matsuura N, Nakajima N. Indocarbazostatin, a novel inhibitor of NGF-induced neurite outgrowth from rat pheochromocytoma PC12 cells. J Antibiot 52: 921–924 (1999)
2. Matsuura N, Tamehiro N, Andoh T, Kawashima A, Ubukata M. Indocarbazostatin and indocarbazostatin B, novel inhibitors of NGF-induced neuronal differentiation in PC12 cells. I. Screening, taxonomy, fermentation and biological activities. J Antibiot 55: 355–362 (2002)
3. Tamehiro N, Matsuura N, Feng Y, Nakajima N, Ubukata M. Indocarbazostatin and indocarbazostatin B, novel inhibitors of NGF-induced neuronal differentiation in PC12 cells. II. Isolation, physicochemical properties and structural elucidation. J Antibiot 55: 363–370 (2002)
4. Kase H, Iwahashi K, Matsuda Y. K-252a, a potent inhibitor of protein kinase C from microbial origin. J Antibiot 39: 1059–1065 (1986)
5. Feng Y, Matsuura N, Ubukata M. Indocarbazostatin C and D, new inhibitors of NGF-induced neuronal differentiation in PC12 cells. J. Antibiot 57: 627–633 (2004)
6. Yasuzawa T, Lida T, Yoshida M, Hirayama N, Takahashi M, Shirahata K, Sano H. The structures of the novel protein kinase C inhibitors K-252a, b, c and d. J Antibiot 39: 1072–1078 (1986)
7. Yang SW, Cordell GA. Biosynthesis of staurosporine: Incorporation of glucose. J Natural Products 59: 826–833 (1996)
8. Kaufman S (*Ed.*). Metabolism of aromatic amino acids and amines. Methods in Enzymology, Vol.142, Academic Press, Inc., Orlando (1987)
9. Meksuriyen D, Cordell GA. Biosynthesis of staurosporine, 2. Incorporation of tryptophan. J Natural Products 51: 44–48 (1988)
10. Williams DE, Bernan VS, Ritacco FV, Maiese WM, Greenstein M, Andersen RJ. Holyrines A and B, possible intermediates in staurosporine biosynthesis produced in culture by a marine actinomycete obtained from the North Atlantic Ocean. Tetrahedron Lett 40: 7171–7174 (1999)
11. Salas AP, Zhu L, Sanchez C, Brana AF, Rohr J, Mendez C, Salas J. A. Deciphering the late steps in the biosynthesis of the anti-tumor indolocarbazole staurosporine: sugar donor substrate flexibility of the StaG glycosyltransferase. Mol Microbiol 58: 17–27 (2005)
12. Onaka H, Asamizu S, Igarashi Y, Yoshida R, Furumai T. Cytochrome P450 homolog is responsible for C–N bond formation between aglycone and deoxysugar in the staurosporine biosynthesis of *Streptomyces* sp. TP-A0274. Bioscie Biotech Biochem 69: 1753–1759 (2005)
13. Wood JL, Stoltz BM, Goodman SN, Onwueme K. Design and implementation of an efficient synthetic approach to pyranosylated indocarbazoles: total synthesis of (+)-RK286c, (+)-MLR-52, (+)-staurosporine, and (−)-TAN-1030a. J Am Chem Soc 119: 9652–9661 (1997)
14. Sanchez C, Butovich IA, Brana AF, Rohr J, Mendez C, Salas JA. The biosynthetic gene cluster for the antitumor rebeccamycin: characterization and generation of indolocarbazole derivatives. Chem Biol 9: 519–531 (2002)
15. Hyun CG, Bililign T, Liao J, Thorson JS. The biosynthesis of indolocarbazoles in a heterologous *Escherichia coli* host. Chembiochem 4: 114–117 (2003)
16. Onaka H, Taniguchi S, Igarashi Y, Furumai T. Cloning of the staurosporine biosynthetic gene cluster from *Streptomyces* sp. TP-A0274 and its heterologus expression in *Streptomyces* lividans. J Antibiot 55: 1063–1071 (2002)
17. Onaka H, Taniguchi S, Igarashi Y, Furumai T. Characterization of the biosynthetic gene cluster of rebeccamycin from *Lechevalieria aerocolonigenes* ATCC 39243. Biosci Biotechnol Biochem 67: 127–138 (2003)